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(57) Abstract

Poly-β-hydroxybutyrate is produced by providing a culture *Escherichia coli* bacterial host cells transformed by a DNA sequence coding for the biosynthetic pathway of poly-β-hydroxybutyrate and a DNA sequence coding for the lysozyme gene; growing the culture and obtaining expression of the poly-β-hydroxybutyrate biosynthetic pathway and the lysozyme gene in each *Escherichia coli* bacterial host cell; lysing the *Escherichia coli* bacterial host cells and collecting the poly-β-hydroxybutyrate. An *Escherichia coli* HMS174(p4A [BstB], pLysS) deposited with the American Type Culture Collection under Accession No. 69001, comprising a plasmid containing a biosynthetic pathway coding for poly-β-hydroxybutyrate and a plasmid containing a lysozyme gene is disclosed.

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DESCRIPTION

METHOD FOR PRODUCTION AND RECOVERY OF POLY-β-HYDROXYBUTYRATE FROM TRANSFORMED *ESCHERICHIA COLI*

TECHNICAL FIELD

The present invention is generally related to the production of poly-beta-hydroxybutyrate (PHB) using Escherichia coli (E. coli) which has been genetically transformed by a vector carrying the genes coding for the PHB biosynthetic pathway and, more particularly, to the more efficient production and recovery of PHB from transformed E. coli.

BACKGROUND ART

PHB is an energy storage material produced by a variety of bacteria in response to environmental stress and is a homopolymer of D-(-)-3-hydroxybutyrate which has properties comparable to polypropylene. Although poly-β-hydroxybutyrate was first described over 60 years ago, the technological potential of the polymer and

related polymers is only now being realized. There is increasing pressure to produce biodegradable plastics due to the problems of waste disposal in general, and disposal of long-lived plastics, specifically. Because PHB is biodegradable, there is considerable interest in using PHB for packaging purposes as opposed to other plastic materials in order to reduce the environmental impact of garbage. PHB also has utility in antibiotics, drug delivery, medical suture and bone replacement applications.

Despite PHB's advantages over other materials, its high cost of production has hindered its performance in the market. The production of the PHB polymer requires extensive treatment after the fermentation phase in order to purify the PHB. This treatment includes mechanical lysis, enzymatic treatment, detergent treatment, washing, agglomeration, and spray-drying. These procedures are expensive and also tend to break down the released granule of PHB.

Currently, two industrial entities are producing PHB. However, the costs of production and recovery of PHB are too high to ensure the use of the plastic as a commodity item. In the first case a regimen of enzymatic treatment and mechanical disruption is used to release the PHB granules from the cells, whereas in the second case, a chlorinated hydrocarbon is used to extract the PHB. In addition, "to the commercial methods currently employed" the related granules can be separated from cell debris by aggregation with salts, such as calcium chloride and other hardening agents, as described in the copending application Serial No. 07/528,549, filed May 25, 1990.

In a copending patent application Serial No. 07/362,514 filed June 7, 1989, and in Slater et al., "Cloning and Expression in Escherichia coli of the Alcaligenes eutrophus H16 Poly-β-Hydroxybutyrate Biosynthetic Pathway", J. Bact., 170:4431, (Oct. 1988), E. coli was genetically transformed with genes from A. eutrophus which code for the PHB biosynthetic pathway. The cloning

of the PHB pathway and its expression in E. coli were also later discussed in Schubert et al., J. Bact. 170:5837 (Dec. 1988), Peoples et al., J. Biol. Chem. 264:15293 (1989a) and Peoples et al., J. Biol. Chem. 264:15298 (1989b). These patent applications and references and all references cited in this disclosure are expressly incorporated herein by reference.

Initial experiments indicated that PHB production in transformed E. coli has not been greater than about 50%, i.e., 30% (Schubert et al., supra.), 50% (Peoples et al., 1989b), 30-54% (Slater et al., supra.) of the bacterial cell weight. However, in a copending patent application to Dennis et al., Serial No. 07/768,008, filed September 27, 1991, improvements have been made to the transformed E. coli PHB production system that result in levels of intracellular PHB as high as 95% of the cell dry weight. PHB accumulation at this level results in a productivity in E. coli (grams produced per liter of culture per hour of time) which is significantly higher than the PHB productivity in A. eutrophus.

There is a need to develop a procedure wherein the PHB granules are gently and efficiently released from the E. coli cell. There is a further need to develop a PHB recovery system which is superior to the existing technology. The result of procedure would be more efficient production of PHB biodegradable plastic products at a greatly reduced cost.

It is therefore an object to the present invention to provide an improved method for producing PHB in transformed E. coli.

It is another object of this invention to provide an improved method for accumulating and recovering PHB from E. coli.

It is another object of this invention to provide transformed E. coli strains which produce high levels of PHB and which can be readily lysed to release such PHB.

DISCLOSURE OF INVENTION

According to the present invention, PHB can be produced at levels of about 90-95% of the cell weight through the use of (1) a gene dosage effect of a plasmid bearing the PHB biosynthetic pathway (such as the p4A plasmid) and/or (2) a "runaway replication" vector containing the PHB biosynthetic pathway. Both types of plasmid vectors accomplish high PHB production by increasing the number of copies of PHB biosynthetic genes in the cell to approximately 500-1000 copies per cell when the cells are grown in Luria broth.

According to the present invention the PHB biosynthetic pathway can be inserted into a runaway replication vector, which is then transferred into a bacterial host such as E. coli. In experiments where the plasmid copy number is increased by heat induction, PHB levels of about 85% to about 90% of the cell weight have been obtained when the construct is grown on Luria broth plus glucose.

Further, according to the present invention, the host bacterial cells are effectively lysed without the need for mechanical action or the addition of enzymatic materials. This is particularly important for the gentle release of PHB granules from the cells. A plasmid containing a lysozyme gene is placed into a bacterial host cell which contains a plasmid bearing the PHB biosynthetic pathway. Thus, the host cells have two plasmids; one plasmid contains the genes for PHB production (optionally a runaway replication vector), and the other plasmid contains the genes for expression of a lysozyme enzyme. The transformed host cell culture is grown for a period of time, during which the PHB granules and the lysozyme enzyme accumulate in the cell. The inner membrane of the host cell does not allow the lysozyme to diffuse through to the peptidoglyan structural or middle layer. At the end of the growth period the inner membrane of the host cell is permeabilized by, for example, exposure to a

permeabilizing agent such as ethylene diamine tetracetic acid (EDTA). This allows the lysozyme to access and weaken the structural peptidoglycan layer located between the inner and outer membranes. For example, if the host cells are resuspended in a solution that contains a (1) an agent such as a nonionic surfactant; and (2) is low in salt and/or if the cells are subjected to repeated freeze-thaw cycles, the weakened bacterial cell ruptures due to osmotic lysis. The granules of PHB are released from the host cell and recovered, by for example, agglomerating the granules with a suitable agglomerating agent.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:

Fig. 1 is a schematic diagram of the plasmid p4A.

Figs. 2A and 2B are schematic diagrams of plasmids pJM9113 and pJM9114, respectively.

Figs. 3A and 3B are schematic diagrams of plasmids pJM9115 and pJM9116, respectively.

Figs. 4A and 4B are schematic diagrams of plasmids pJM9117 and pJM9118, respectively.

Figs. 5A and 5B are schematic diagrams of plasmids pJM9119 and pJM9120, respectively.

Figs. 6A and 6B are schematic diagrams of plasmids pJM9125 and pJM9126, respectively.

Figs. 7A and 7B are photographs showing lysis of PHB from clones having the (A) pLysS gene in the S4 strain; and, (B) pLysE gene in the E5 strain.

Figs. 8A, 8B and 8C are graphs showing the growth curves, optical density (600 nm) and PHB accumulation (mg/ml) over time grown on glucose and on whey of (A) strain HMS174/p4A, (B) S4 and (C) E5.

Fig. 9 is a graph showing the isopycnic centrifugation of lysed S4 cells, optical density (600 nm) and sucrose concentration.

Fig. 10 is a schematic diagram of the plasmid pJM9101.

Figs. 11A and 11B are photographs of (A) PHB production in TRED1.9 cells, and (B) lysis of TRED1.9.

Figs. 12A, 12B and 12C are photographs of S4 cells that have been lysed by the disruption of their polar caps.

Fig. 13 is a photograph of aggregated granules of PHB.

Fig. 14A is a photograph of aggregated granules of PHB.

Fig. 14B is a photograph of aggregated granules of PHB using 10 mM calcium chloride as an agglomerating agent.

Fig. 14C is a photograph of aggregated granules of PHB using 100 mM calcium chloride as an agglomerating agent.

Fig. 15A is a graph showing the optical density O.D. (600nm) of E. coli HMS174 (pJM9125) over time.

Fig. 15B is a graph of a fermentation experiment showing PHB content (%) ■, dry biomass (conc)(g/L)○, and PHB (conc)(g/L)□.

BEST MODE OF CARRYING OUT INVENTION

The present invention advantageously provides for increased production of poly-β-hydroxybutyrate (PHB) using E. coli which has been genetically transformed by a vector carrying the genes coding for the PHB biosynthetic pathway.

The cloning of the PHB pathway and its expression in E. coli was first disclosed and claimed in the copending application Serial No. 07/362,514 and disclosed in the Slater et al., supra, article. The "first generation" clones consisted of a 5.2 kb *Kpn*I-*Eco*RI fragment

containing the PHB biosynthetic pathway cloned into the vectors pTZ18U-PHB (United States Biochemicals) or pGEM7f+ (Promega Biotec). These clones have approximately 800 bases of non-essential information to the upstream side of the pathway, and 400 bases of non-essential information to the downstream side. In various experiments, these clones produced PHB to levels reaching approximately 50% of the total cell weight. A specific clone that outperforms these clones by a considerable margin is shown in Fig. 1. This clone, designated p4A, is a derivative of pTZ18U-PHB in which approximately 400 bases of the insert DNA (PHB genes) have been deleted starting at the unique *Hind*III site. The p4A plasmid has been placed in several different E. coli strains, including DHI, DH5, BW313, HMS174 and CJ236. In all instances PHB was produced at levels of about 70-95% PHB wt/cell wt.

The p4A plasmid was constructed from pTZ18U-PHB which was constructed from pSB20, which was constructed from pBK12 plasmids containing the PHB biosynthetic pathway. The pBK12 and pSB20 plasmids were disclosed in the copending patent application, Serial No. 07/362,514, filed June 7, 1989. The p4A plasmid was disclosed in the copending patent application 07/528,549 filed May 25, 1990.

Clone (plasmid) p4A contains the genes for the poly-β-hydroxybutyrate biosynthetic pathway and was deposited in an E. coli HMS174 host in the permanent collection of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, on May 23, 1990 and assigned the accession number ATCC 68329. This deposit is available to the public upon the grant of a patent disclosing it. The deposit is also available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the

subject invention in derogation of patent rights granted by governmental action.

The present invention also advantageously provides that the PHB biosynthetic genes can be obtained from any of the aforementioned plasmids, or subclones thereof, and inserted into an expression vector such as a runaway replication plasmid vector in order to have even greater expression of the PHB polymer. Runaway replication plasmid vectors induce uncontrolled replication (runaway replication) by a simple temperature shift or a change in media constituents, which leads to an increase in plasmid copy number from a few per genome to as much as a thousand or more per genome over a period of only 4-6 cell generations.

Runaway replication plasmid vectors are useful for expressing PHB since PHB has a negative effect on cell growth. When the cell culture is at low density and runaway replication has not been induced, the PHB gene is present in low dosage and expression of PHB is minimized. After high cell density has been reached, runaway replication is induced causing an exponential increase in copy number and gene dosage. The combination of high cell density and rapidly increased gene dosage results in high yield expression of PHB in the relatively short time period before cell growth stops. The PHB biosynthetic genes have been placed into various runaway replication vectors.

A series of new constructs is disclosed herein in which the PHB pathway has been inserted into the runaway replication vectors pRA87, pRA88, pRA89, and pRA90 which were obtained from the Nykomed Pharma Co. of Copenhagen, Denmark. The new constructs are made by inserting the PHB biosynthetic pathway into the plasmids named above. The resulting plasmids are shown in Figs. 2-6.

The plasmids pJM9113 and pJM9114, as shown in Figs. 2A and 2B, respectively, contain the PHB pathway cloned in opposite

orientation into the *Bam*HI site of pRA87. The plasmids pJM9113 and pJM9114 are induced into runaway replication above 37°C and have a basal copy number of 10 at 30°C. Thus, pJM9113 and pJM9114 are both plasmids derived from pRA87, wherein the PHB pathway is in a different orientation in each plasmid.

The plasmids pJM9115 and pJM9116, as shown in Figs. 3A and 3B, respectively, contain the PHB pathway cloned in opposite orientation into the *Bam*HI site of pRA88. The pRA88 plasmid is resistant to chloramphenicol at 30 μg/ml, and has cloning sites *Bam*HI, *Sac*I and *Kpn*I. Thus, pJM9115 and pJM9116 are both plasmids derived from pRA88, wherein the PHB pathway is in a different orientation in each plasmid. The plasmids pJM9115 and pJM9116 are induced into runaway replication at 37°C and have a basal copy number of 10 at 30°C. When the pJM9116 plasmid is inserted into the E. coli HMS174 host cells, PHB is produced at levels of approximately 85% PHB (by GC analysis) of dry cell weight.

The plasmids pJM9117 and pJM9118, shown in Figs. 4A and 4B, respectively, contain the PHB pathway cloned in opposite orientation into the *Bam*HI site of pRA89. The plasmid pRA89 cloning sites include *Bam*I, *Sac*I and *Kpn*I. Thus, pJM9117 and pJM9118 are both derived from pRA89 wherein the PHB pathway is in a different orientation in each plasmid. The plasmids pJM9117 and pJM9118 are induced into runaway replication at 41°C and have a basal copy number of 1.

The plasmids pJM9119 and pJM9120, as shown in Figs. 5A and 5B, respectively, contain the PHB pathway cloned in opposite orientation into the *Bam*HI site of pRA90. The pRA90 is resistant to chloramphenicol at 30μg/mL and has cloning sites at *Bam*HI, *Sac*I and *Kpn*I. Thus, pJM9119 and pJM9120 are both derived from pRA90 wherein the PHB pathway is in a different orientation in each plasmid.

The plasmids pJM9119 and pJM9120 are induced into runaway replication at 41°C and have a basal copy number of 1.

The plasmids pJM9125 and pJM9126, as shown in Figs. 6A and 6B, respectively, contain the *sup*F gene of E. coli cloned into a *Bam*HI site of the pJM9116 plasmid. The *sup*F gene encodes a transfer RNA that suppresses certain amber mutations and is useful in stabilizing the pJM9125 and pJM9126 plasmids in E. coli cells containing a dna*Bam* mutation. dnaB codes for the DNA helicase and is absolutely essential for cell growth. In the absence of the suppressor gene (supF) on the plasmid the amber mutation in dnaB would be a lethal mutation, but as long as the plasmid is present, it supplies the necessary suppressor tRNAs to negate the amber mutation in the helicase. On the other hand, if the plasmid is lost, the cell cannot replicate and eventually dies. In the pJM9125 plasmid, the *sup*F gene is cloned into the *Bam*HI site at the end of the PHB pathway. In the pJM9126 plasmid, the *sup*F is cloned into the *Bam*HI site at the end of the PHB pathway in opposite orientation from the pJM9125 plasmid. The plasmids pJM9125 and pJM9126 are induced into runaway replication at 37°C and have a basal copy number of 10. When the pJM9125 and pJM9126 plasmids are inserted into the E. coli HMS174 host cells, PHB is produced at levels of approximately 90% PHB (GC analysis) of dry cell weight.

E. coli hosts containing the plasmids of the subject invention were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, USA. The accession numbers and deposit dates are as follows:

Culture containing	Accession Nos.	Deposit date
pJM9101	ATCC 69000	May 21, 1992
pJM9113	ATCC 68989	May 21, 1992
pJM9114	ATCC 68990	May 21, 1992
pJM9115	ATCC 68991	May 21, 1992

pJM9116	ATCC 68992	May 21, 1992
pJM9117	ATCC 68993	May 21, 1992
pJM9118	ATCC 68994	May 21, 1992
pJM9119	ATCC 68995	May 21, 1992
pJM9120	ATCC 68996	May 21, 1992
pJM9125	ATCC 68998	May 21, 1992
pJM9126	ATCC 68999	May 21, 1992

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of the deposits does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposits, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

The present invention also advantageously provides for increased recovery of PHB. To retrieve the purified PHB produced in

transformed E. coli in large quantities, the transformed E. coli cells are lysed by genetic means. According to one embodiment, a plasmid containing a lysozyme gene is placed in the E. coli bacterial host cells which contain the PHB biosynthetic pathway on a separate, compatible plasmid. In another embodiment the lysozyme gene is inserted into the plasmid carrying the genes coding for PHB. It is also contemplated that other useful genes, including, for example, a stabilization gene, can also be inserted into the plasmid carrying the PHB coding genes. As the cell grows, more and more lysozyme is made.

Normally, lysozyme cannot cross the inner lipopolysaccharide membrane of the host cell and degrade the middle structural membrane of the cell (peptidoglycan). The lysozyme plasmid causes lysozyme to be continuously made during the growth of the cell. It does not, however, "weaken the cell wall" during the growth period. If it did, the cells could not divide. Instead, the lysozyme is accumulated inside the cell. The inner membrane does not allow the lysozyme to diffuse through to the peptidoglycan layer. As long as the lysozyme is denied access to the peptidoglycan layer by the inner membrane, it does not weaken the cell wall. The presence of the lysozyme plasmid (which is compatible) stabilizes the PHB plasmid and allows more PHB production than normal. At the end of the growth cycle, when the maximum amount of PHB is made, the cells are induced to lysis, for example, in a manner as described in Studier et al. Methods in Enzymology 185:60-89 (1990) wherein an agent is added to the cell that permeabilizes (makes holes in) the inner membrane. These agents include, for example, ethylene diamine tetra acetic acid (EDTA). It should be understood that various other permeabilizing agents can be utilized in the present invention.

Once the inner membrane is broken down, the lysozyme starts to weaken the cell wall. Therefore, it is possible to control the time

at which the cell wall is weakened. Once the cell wall is weakened, the cells can be lysed by the addition of a suitable nonionic surfactant. One suitable nonionic surfactant comprises Triton® X-100 (octyl phenoxy polyethoxy ethanol) at a concentration ranging from about 0.01% to about 1.0%. A particularly suitable concentration of Triton® X-100 is about 0.1%. It is also contemplated that the cells can be lysed by repeated cycles of freeze-thawing of the cells. The ends of the bacterial cells are destroyed and the PHB granules are extruded out of the ends of the cells. In a preferred embodiment the cells are pelleted by centrifugation, washed, and then resuspended in a permeabilizing agent, such as 50 mM Tris/2 mM EDTA (pH 8) or other suitable agent. The presence of the EDTA allows intracellular lysozyme to weaken the cell wall. A surfactant can be added in a preferred embodiment to a final concentration of 0.1%, to cause almost complete lysis of all the cells thereby releasing the PHB granules.

While the granules of PHB tend to self-aggregate, this aggregation can be enhanced by adding an agglomerating agent. The aggregated PHB granules are then collected. According to the present invention, E. coli cells can accumulate PHB to approximately 90-95% of the cell weight, and there is relatively little purification needed after the collection of the PHB, other than washing of the PHB granules.

The various methods employed in the preparation of the plasmids and transformation of host organisms are described in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.

The restriction enzymes disclosed can be purchased from various commercially available sources. The enzymes are used according to the instructions provided by the supplier.

Two types of assay were used for PHB quantification. In most instances, the procedure of Braunegg et al., Euro. J. Appl. Microbio. and Biotech. 6:29 (1978) was used. When necessary to check the data for precision, the procedure of Law et al., J. Bact., 82:33 (1961) was also used.

The following examples are provided to illustrate certain preferred embodiments of the present invention, and are not restrictive of the invention, as claimed.

Example 1

The following is an example of electroporation of lysozyme plasmids, pLysS and pLysE, into E. coli HMS174(p4A) [Bst B-].

E. coli HMS174(p4A) is a strain carrying the p4A plasmid. The restriction enzyme site BstB1, has been deleted from of the plasmid. E. coli HMS174 was grown to mid-logarithmetic phase and prepared for electroporation using standard techniques as described in Maniatis, et al. supra. The plasmids pLysS and pLysE, which encode the T7 bacteriophage lysozyme enzyme, were isolated from bacterial strains provided by Brookhaven National Laboratory, Upton, Long Island, New York. The pLysS and pLysE plasmids were co-electroporated into the above cells in two separate electroporations. That is, pLysS and p4A (BstB-) were used together, and pLysE and p4A (BstB-) were used together. Electroporated cells were plated into Luria agar plates containing ampicillin, chloramphenicol, and tetracycline. These antibiotics select for the BstB- plasmid, the pLysS and pLysE plasmid, and the HMS174 phenotype, respectively. Four transformants from the pLysS electroporation (designated S1 thru S4) were selected and grown overnight in Luria broth + 1% glucose. Five colonies from the pLysE electroporation were selected and also grown as above (designated E1 thru E5). The cultures were grown approximately 24 hours at 37°C on an orbital shaker incubator. The

optical density at 600 nm of each culture was measured, and the relative amount of PHB was measured by dissolving the cellular material with Clorox® (sodium hypochlorite) and reading the resultant optical density of PHB granules at 600 nm. As can be seen in the Table I below the transformants labeled S2, S4 and E5 had very good PHB production.

TABLE I

Transformants	Optical Density (λ600.0)
Cells diluted 1:10	
S1	1.172
S2	1.346
S3	1.204
S4	1.327
E1	0.450
E2	0.391
E3	0.323
E4	0.708
E5	1.082
Cells exposed to Clorox® 1 hr., diluted 1:10	
S1	1.096
S2	1.145
S3	1.037
S4	1.208
E1	0.163
E2	0.088
E3	0.044
E4	0.352
E5	1.013

The strain S4, designated as E. coli HMS174(p4A [BstB],pLysS) was deposited in accordance with the terms of the Budapest Treaty with the American Type Culture Collection, 1230 Parklawn Drive, Rockville, MD, under Accession No. 69001, on May 21, 1992. This deposit was made under conditions as provided under ATCC's agreement for Culture Deposit for Patent Purposes, which assures that the deposit will be made available to the U.S. Commissioner of Patents and Trademarks pursuant to 35 USC 122 and 37 CFR 1.114, and will be made available to the public upon issue of a U.S. patent, which requires that the deposit is available to the public upon the grant of a patent disclosing it. The deposit is also available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Example 2

The following is an example of lysis of PHB from E. coli HMS174(p4A [BstB-]).

The three transformants in Example 1 above were found to have the greater PHB production (S2, S4 and E5) and were grown in Luria broth containing 1% glucose, 25 μg/ml ampicillin, and 34 μg/ml chloramphenicol for 38 hours at 37°C (3 ml cultures in 16 x 100 mm culture tubes). The cells were pelleted by centrifugation, frozen, and then resuspended in 50 mM Tris/2 mM EDTA. Triton® X-100 surfactant was added to 0.1% final concentration. Samples of cells from each strain were placed on microscope slide and examined for lysis. The best clones for lysis were S4 and E5. Figs. 7A and 7B show these clones after they have accumulated PHB and have been lysed.

Example 3

The following is an example of the growth and PHB production the S4 and E5 transformants to determine whether the S4 and E5 lysis strains are as proficient at producing PHB as nonlysis strains.

The three strains which were compared are:

E. coli HMS174(p4A)

E. coli HMS174(p4A [BstB-], (pLysS) (isolate S4)

E. coli HMS174(p4A[BstB-](pLysE) (isolate E5)

Cultures were grown for approximately 2 days in both glucose and whey (separate cultures). At specified time points, the optical density at 600 nm (rough measure of cell density), and the PHB content (by gas chromatography) was analyzed. PHB production in the lysis strains was compared with PHB producer, E. coli HMS174(p4A). The results shown in Figs. 8A, 8B and 8C show that the S4 strain (Fig. 8B) produced PHB as well as the E. coli HMS174(p4A) strain (Fig. 8A).

Example 4

The following is an example which shows that PHB granules can be removed from the cell debris after lysis by isopycnic gradient centrifugation. The S4 strain was cultured for 2 days under conditions that promote PHB synthesis. The culture was lysed and loaded onto an isopycnic gradient. The gradient was run for 4 hours at 25,000 rpm at 20°C. The gradient was fractionated and fractions were analyzed for PHB content, optical density at 600 nm, and density. The graph shown in Fig. 9 shows two major peaks, one at fractions 40-41, and the other at fraction 44. These correspond to PHB granule and cell debris, respectively.

Example 5

An E. coli HMS174 strain (Tred1.9) was developed which contains the plasmid, pJM9101 as shown in Fig. 10. (The strain Tred1.9 is the S4 strain in which the p4A plasmid has a kanamycin resistance gene inserted.) The pJM9101 plasmid contains the p4A that has a kanamycin resistance gene inserted at the *Eco*RI site at the end of the PHB pathway. The strain Tred1.9 was made by placing pJM9101 and pLysS together in an HMS174 E. coli. The strain was tested for lysis proficiency. The strain was grown overnight in 3 ml culture containing kanamycin and chloramphenicol. Cells were harvested by pelleting, resuspended in 50 mM Tris/2 mM EDTA and Triton® X-100 surfactant was added to a final concentration of 0.1%. Lysis, as monitored by light microscopy was nearly 100% complete. Very good PHB production can be seen in the photograph of Fig. 11A. Good lysis and aggregation (arrows) can be seen in the photograph of Fig. 11B.

Example 6

The lysed cells of Example 3 were examined using high magnification microscopy. Standard fixation and electron microscopic techniques were used on S4 cells that had been lysed. The cell lysate mixture was collected on a 0.22 nm membrane filter. The filter was prepared for scanning electron microscopy. The photographs shown in Figs. 12A, 12B and 12C show aggregated PHB granules that have been released from lysed cells. Fig. 12A shows that the granules are released and tend to self-aggregate. It can also be seen that the cells have been lysed by the disruption of their polar caps. What is left is a small tube with holes at each end. Fig. 12B is a magnification of one of the tubes showing that the polar cap has been "blown off". Fig. 12C is a magnification of the aggregated granules.

Example 7

While the granules tend to self-aggregate, the aggregation or agglomeration can be accelerated and controlled by use of an agglomerating agent. Aggregation can be further enhanced by varying the concentration of the agglomerating agent.

Suitable agglomeration agents have been disclosed in the copending application Serial No. 07/528,549, and include, for example, KH_2PO_4 (potassium phosphate-monobasic), NaCl (sodium chloride), $MgSO_4$ (magnesium sulfate), K_2HPO_4 (potassium phosphate-dibasic), $MgCl_2$ (magnesium chloride), $(NH_4)_2HPO_4$ (ammonium phosphate-dibasic), MgOAC (magnesium acetate), NaOAC (sodium acetate), $CaCl_2$ (calcium chloride). Fig. 13 shows a scanning microphotograph of PHB granules aggregated by using calcium chloride. The aggregation of PHB granules is enhanced by increasing the concentration of agglomerating agent. Fig. 14A shows the aggregation of PHB without any agglomerating agent. Fig. 14B shows the aggregation of PHB in the presence of 10 mM calcium chloride as the agglomerating agent. Fig. 14C shows the aggregation of PHB in the presence of 100 mM calcium chloride. It can be seen that the aggregation of PHB is greater using 10 mM calcium chloride and that 100 mM calcium chloride produces even greater aggregation of PHB.

Example 8

The following is an example showing the PHB production in an E. coli HMS174 containing the plasmid pJM9125. Referring now to Figs. 15A and 15B, a maximum O.D. of over 200 was attained after 36 hours corresponding to biomass concentrations of up to about 61 g/L. A maximum PHB concentrate of 46.4 g/L was achieved after only 28 hours with a PHB content of 88.2% (corresponding to a total biomass concentration of 52.6 g/L and O.D. of 164). After 28 hours

the PHB content dropped sharply. Thus, all the subsequent increase in O.D. and biomass concentration was not associated with PHB production. The PHB yield coefficient at 28 hours was 0.35. The maximum PHB productivity was 1.66 g/L.h The PHB content for the E. coli HMS174(pJM9125) ranged from about 72.6% to about 88.2%.

The descriptions of the foregoing embodiments of the invention have been presented for the purpose of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

CLAIMS:

1. A process for producing poly-β-hydroxybutyrate comprising:

providing a culture of Escherichia coli bacterial host cells transformed by (1) an isolated DNA sequence coding of biosynthetic pathway of poly-β-hydroxybutyrate, and (2) an isolated DNA sequence of a lysozyme gene;

growing the culture in a suitable medium and obtaining expression of the poly-β-hydroxybutyrate biosynthetic pathway and the lysozyme gene in each Escherichia coli bacterial host cell;

isolating the poly-β-hydroxybutyrate by lysing the Escherichia coli bacterial host cells; and,

collecting the poly-β-hydroxybutyrate.

2. The process of claim 1 in which the Escherichia coli bacterial host cells are lysed by exposing the cells to a permeabilizing agent of sufficient strength to permeabilize the host cell's inner membrane.

3. The process of claim 2, wherein the host cells are further exposed to a suitable surfactant.

4. The process of claim 1 in which the Escherichia coli bacterial host cells are lysed by pelleting the cells and resuspending the cells in a permeabilizing agent.

5. The process of claim 1 in which the Escherichia coli bacterial host cells are lysed by pelleting the cells, resuspending the cells in a permeabilizing agent, and thereafter, freezing the cells.

6. The process of claim 1 in which the Escherichia coli bacterial host cells comprise Escherichia coli HMS174.

7. The process of claim 1 in which the DNA sequence coding for the poly-β-hydroxybutyrate biosynthetic pathway comprises a DNA sequence from Alcaligenes eutrophus.

8. The process of claim 7, in which the DNA sequence coding for poly-β-hydroxybutyrate comprises p4A plasmid.

9. The process of claim 1, in which the DNA sequence coding for the lysozyme gene is obtained from a T7 bacteriophage.

10. The process of claim 1 which the Escherichia coli bacterial host cells are further transformed by a stabilization gene.

11. The process of claim 10 in which the stabilization gene comprises the *sup*F gene.

12. The process of claim 7, in which the transformed Escherichia coli is transformed with a plasmid selected from the group consisting of pJM9101, pJM9113, pJM9114, pJM9115, pJM9116,

pJM9117, pJM9118, pJM9119, pJM9120, pJM9125, and pJM9126.

13. The process of claim 1 in which the Escherichia coli bacterial host is transformed by a vector having a DNA sequence coding for poly-β-hydroxybutyrate and a DNA sequence coding for a lysozyme enzyme.

14. The process of claim 1 in which the Escherichia coli bacterial host comprising a strain (S4) designated as E. coli HMS174(p4A [BstB-],pLysS), deposited with the American Type Culture Collection under ATCC Accession No. 69001.

15. The process of claim 1 in which poly-β-hydroxybutyrate is produced at levels of about 90 to about 95%, by weight, of dry cell weight.

16. The process of claim 1 in which the isolated DNA sequence of the biosynthetic pathway of poly-β-hydroxybutyrate is contained on a high copy number plasmid.

17. An Escherichia coli, which has been transformed with Alcaligenes eutrophus genes coding for a biosynthetic pathway of poly-β-hydroxybutyrate and with genes coding for a lysozyme enzyme, wherein the genes are expressed at sufficient levels to confer upon the Escherichia coli transformant the ability to produce poly-β-hydroxybutyrate.

18. The Escherichia coli, according to claim 17, wherein the Escherichia coli has been transformed with a plasmid selected from the group consisting of pJM9101, pJM9113, pJM9114, pJM9115,

pJM9116, pJM9117, pJM9118, pJM9119, pJM9120, pJM9125, and pJM9126.

19. The Escherichia coli, according to claim 17, wherein the Escherichia coli has been transformed with pJM9126.

20. An Escherichia coli bacterial host HMS174(p4A [BstB-], pLsyS) strain (S4), deposited with the American Type Culture Collection under Accession No. 69001.

21. Poly-β-hydroxybutyrate produced by:

providing a culture of Escherichia coli bacterial host cells transformed by (a) an isolated DNA sequence of a biosynthetic pathway of poly-β-hydroxybutyrate; and (b) an isolated DNA sequence of a lysozyme gene;

growing the culture and obtaining expression of the poly-β-hydroxybutyrate biosynthetic pathway and the lysozyme gene in each Escherichia coli bacterial host cell;

isolating the poly-β-hydroxybutyrate by lysing the Escherichia coli bacterial host cells; and,

collecting the poly-β-hydroxybutyrate.

22. A method for the production of poly-β-hydroxybutyrate, the method comprising the transforming an Escherichia coli with (1) Alcaligenes eutrophus genes coding for a biosynthetic pathway of poly-β-hydroxybutyrate and (2) a stabilization gene, wherein the genes are expressed by the transformed Escherichia coli at sufficient levels to result in the production of poly-β-hydroxybutyrate as a fermentation product when the Escherichia coli is grown in an appropriate medium.

23. The method according to claim 22, wherein the Escherichia coli is transformed with a plasmid selected from the group consisting of pJM9101, pJM9113, pJM9114, pJM9115, pJM9116, pJM9117, pJM9118, pJM9119, pJM9120, pJM9125 and pJM9126.

24. The method according to claim 23, wherein the Escherichia coli has been transformed with pJM9126.

25. The method according to claim 22, wherein a p4A plasmid contains the Alcaligenes eutrophus genes coding for the poly-β-hydroxybutyrate biosynthetic pathway.

26. The method according to claim 22, wherein the stabilization gene comprises a *sup*F gene.

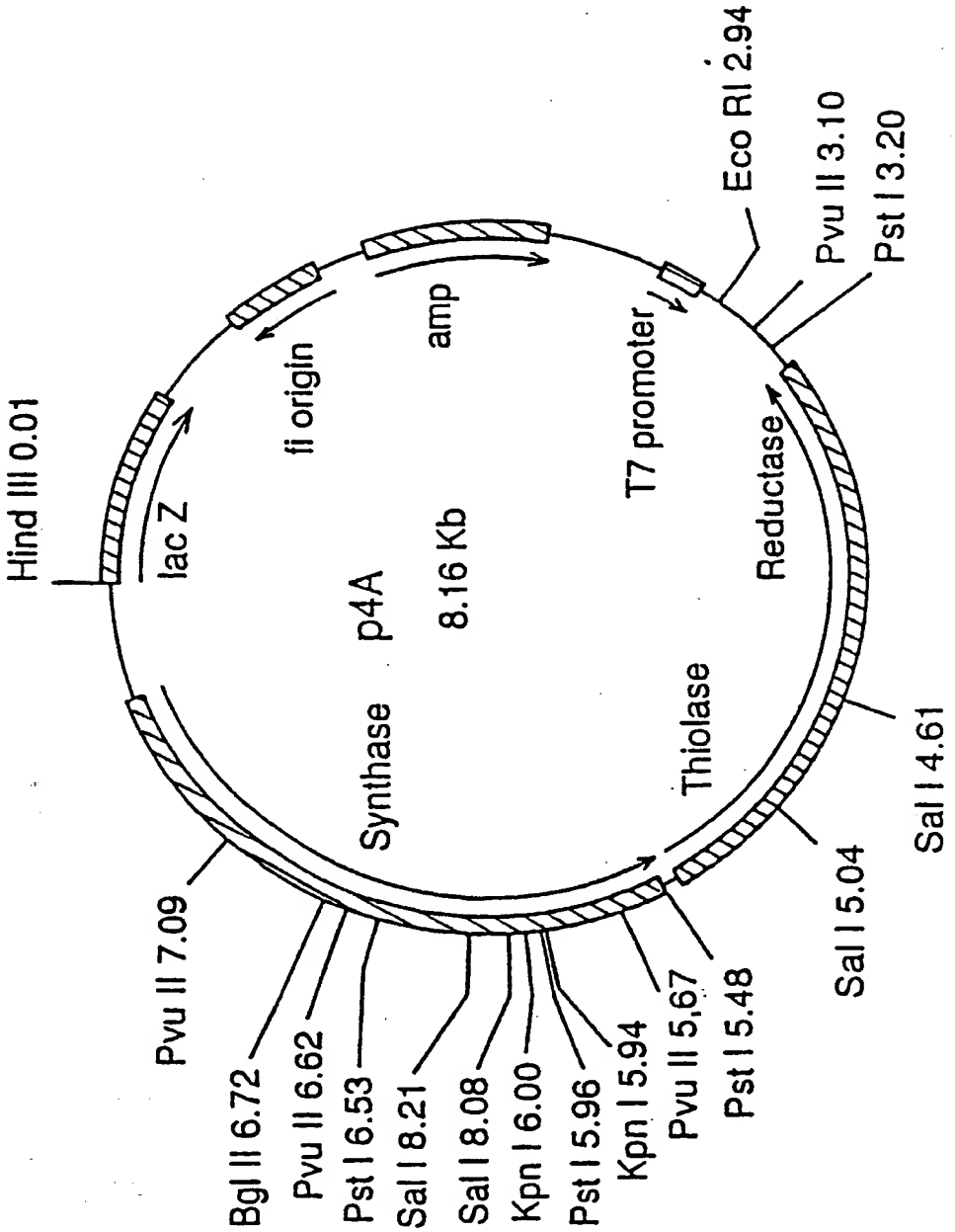


FIG. 1

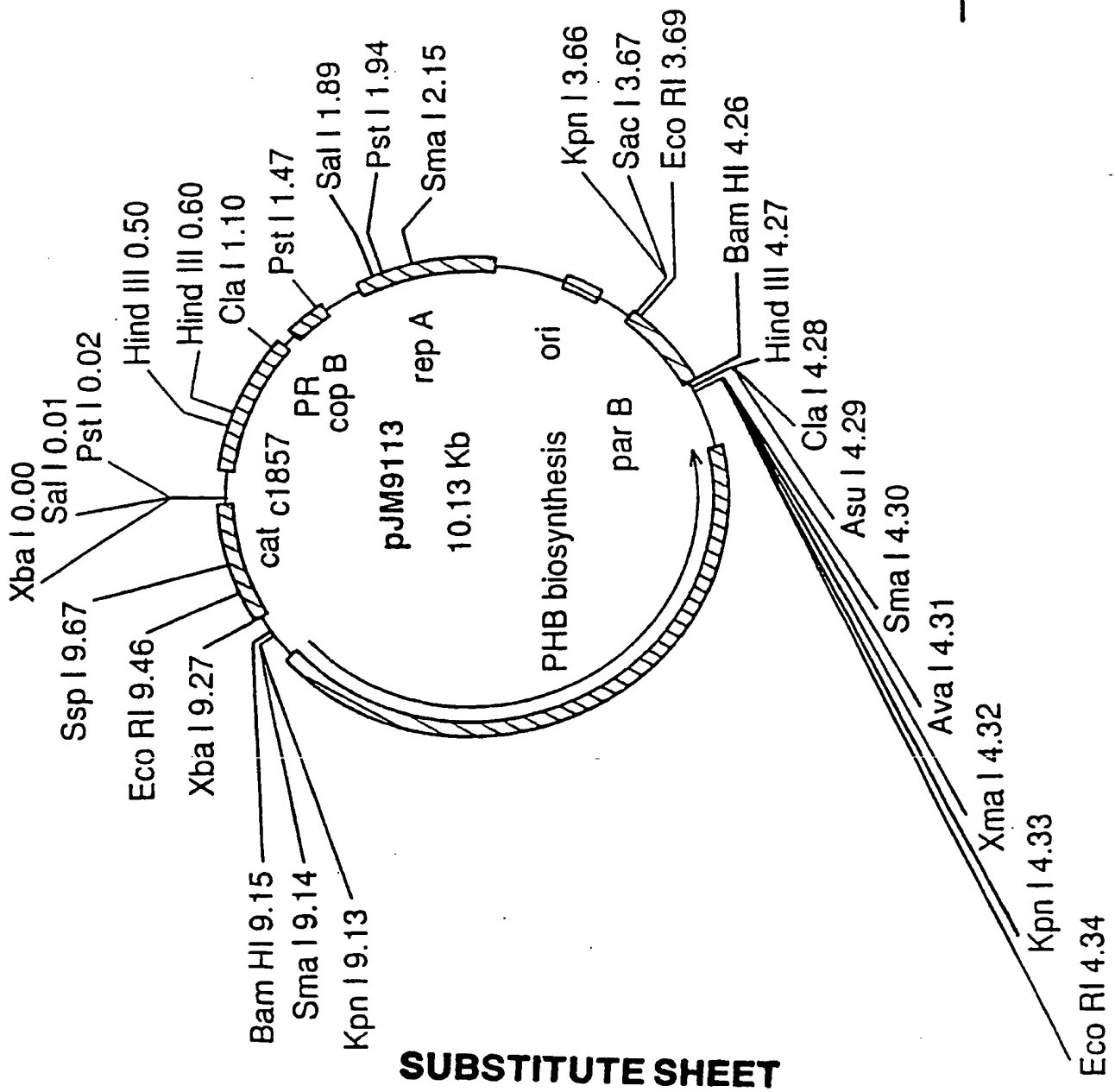


FIG. 2A

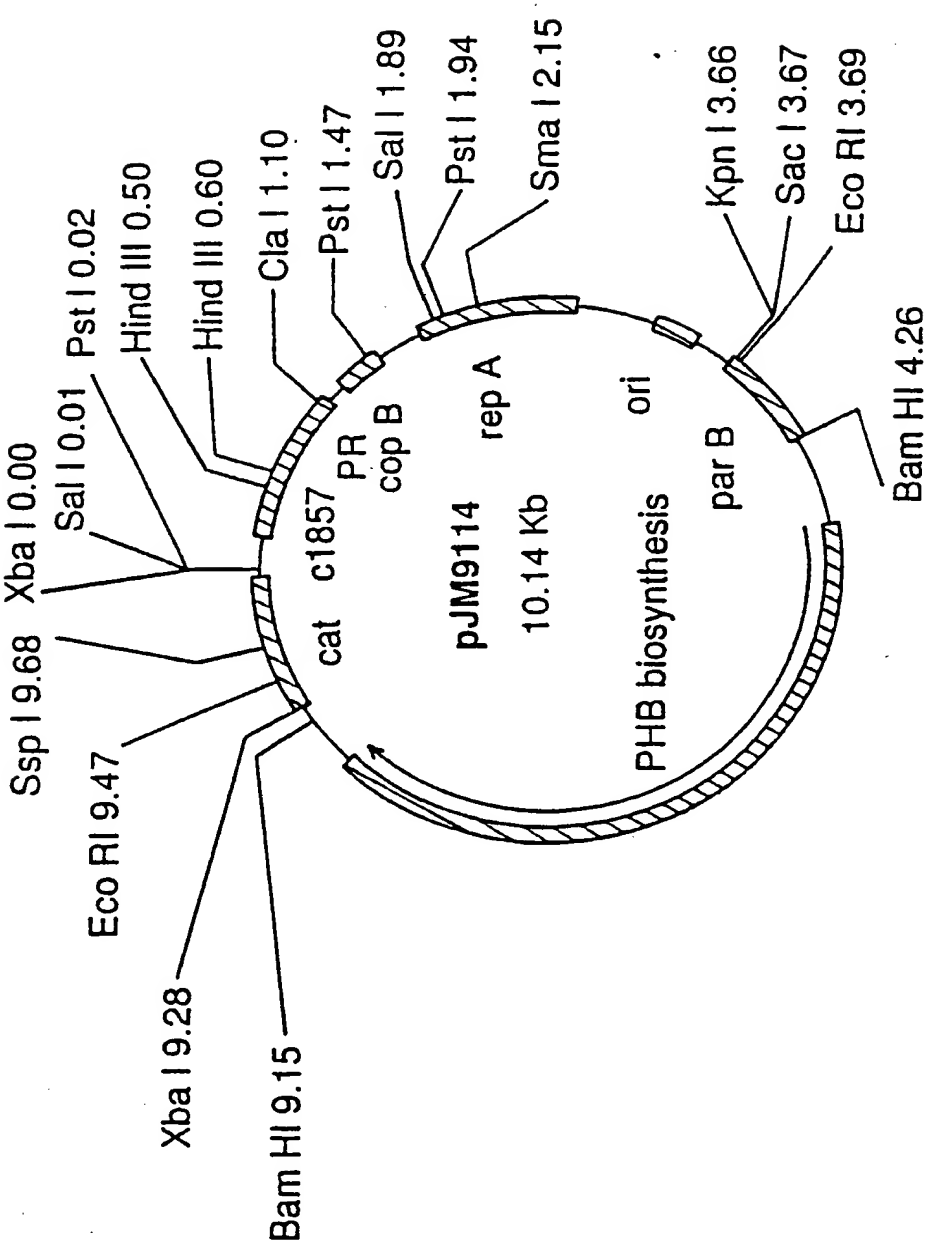


FIG. 2B

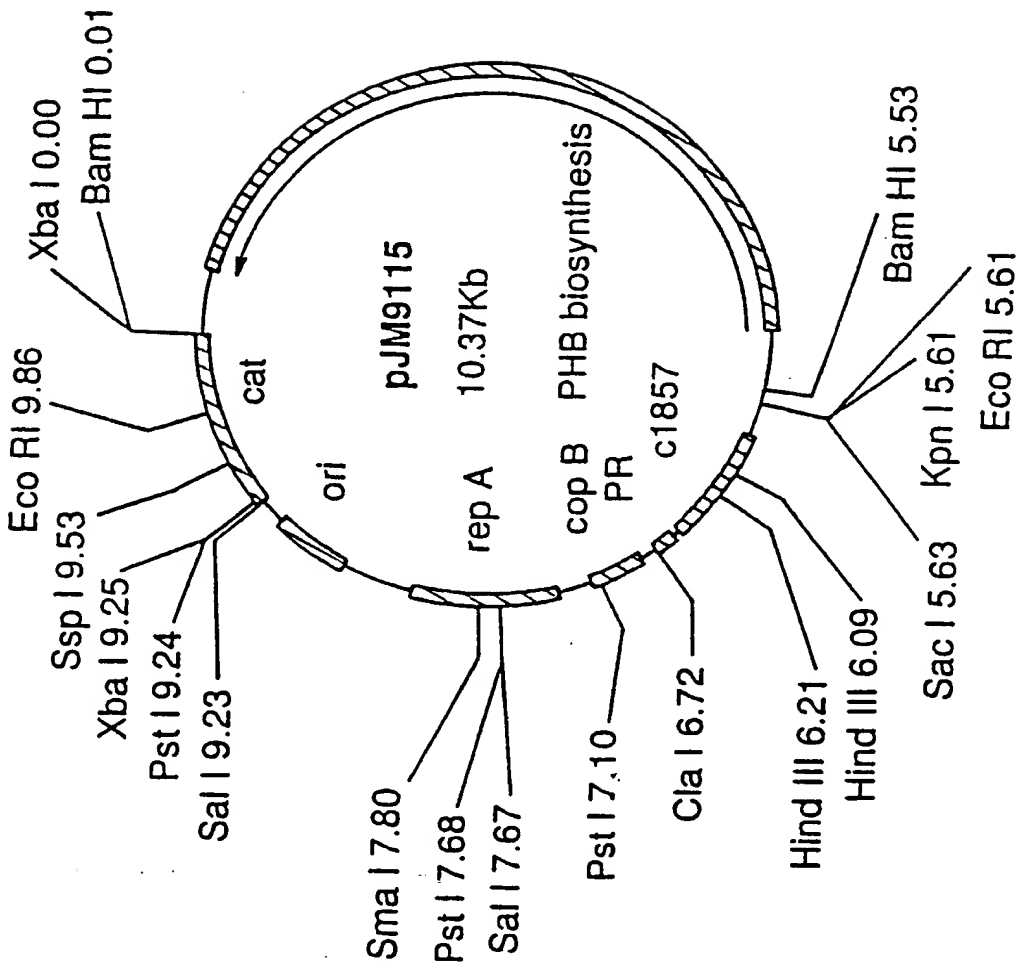


FIG. 3A

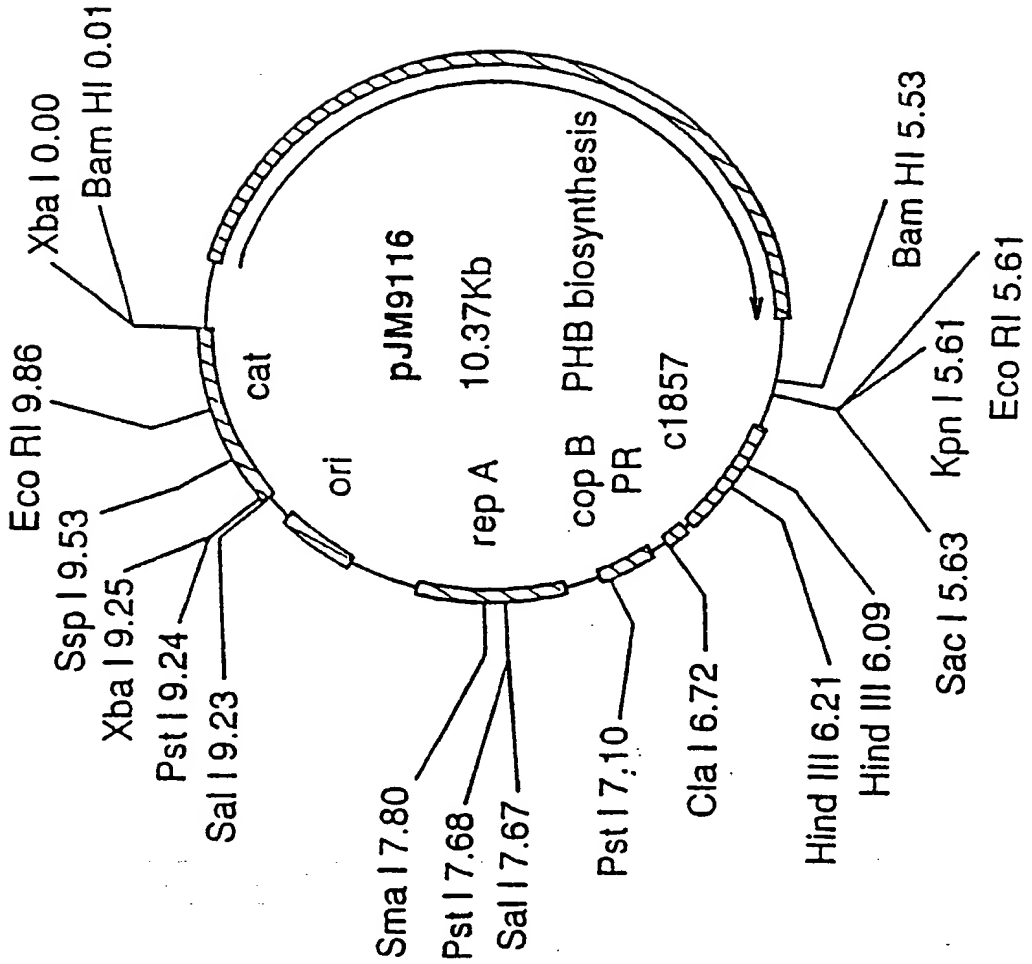


FIG. 3B

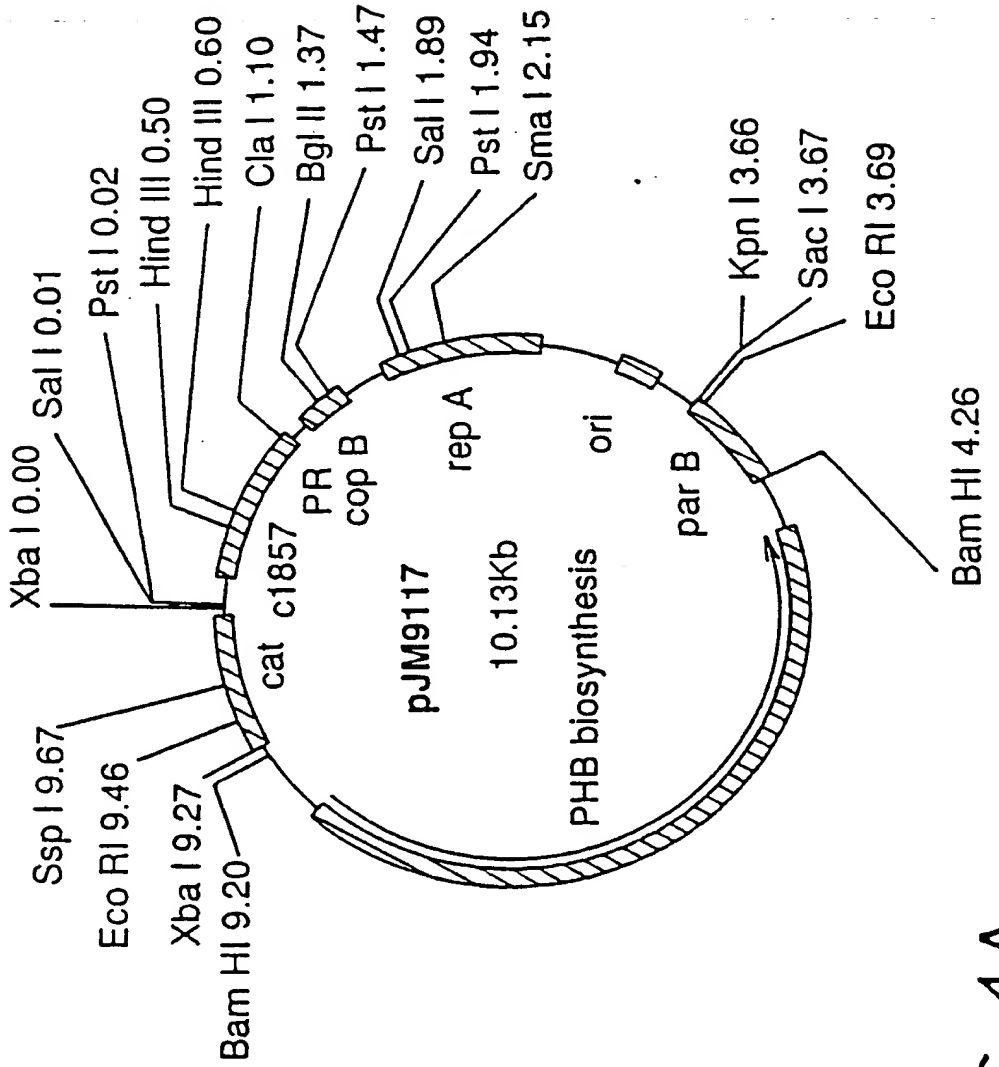


FIG. 4A

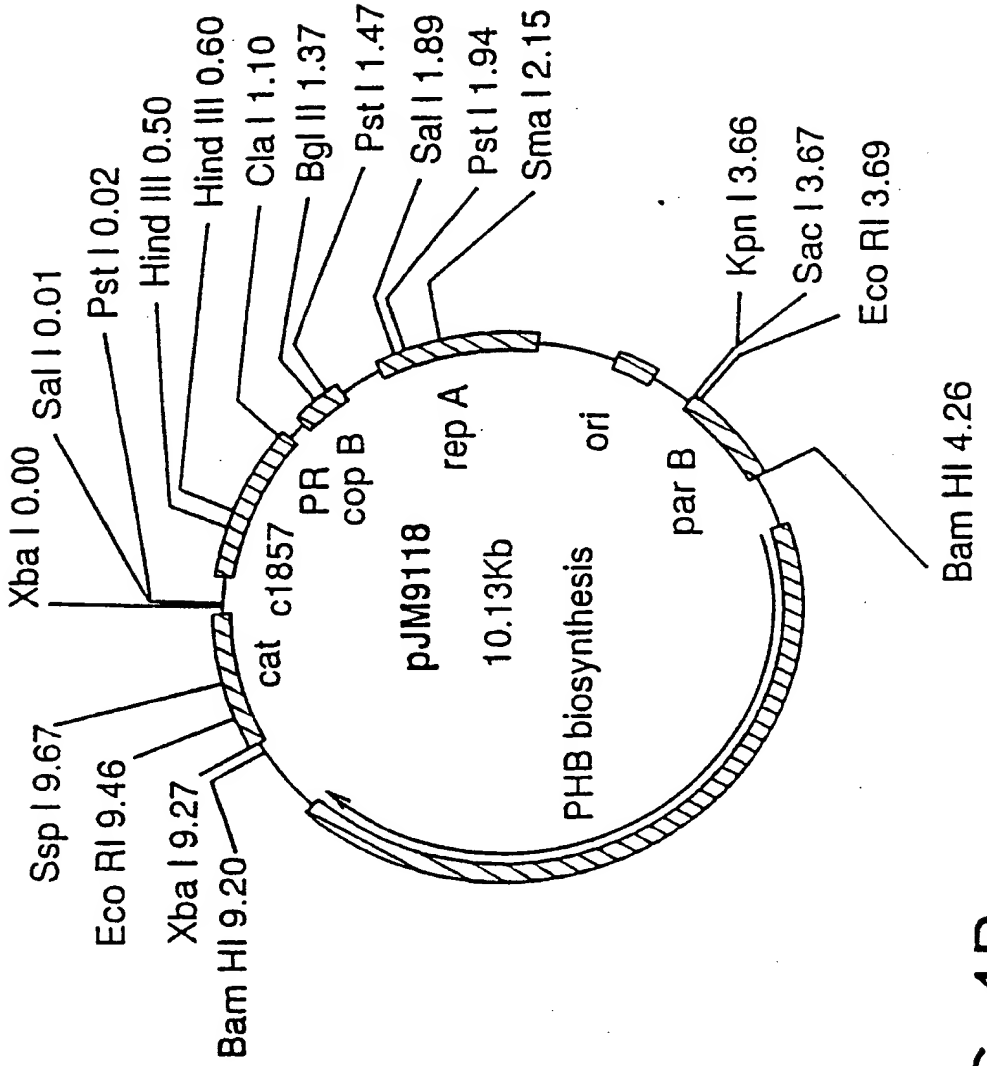


FIG. 4B

FIG.5A

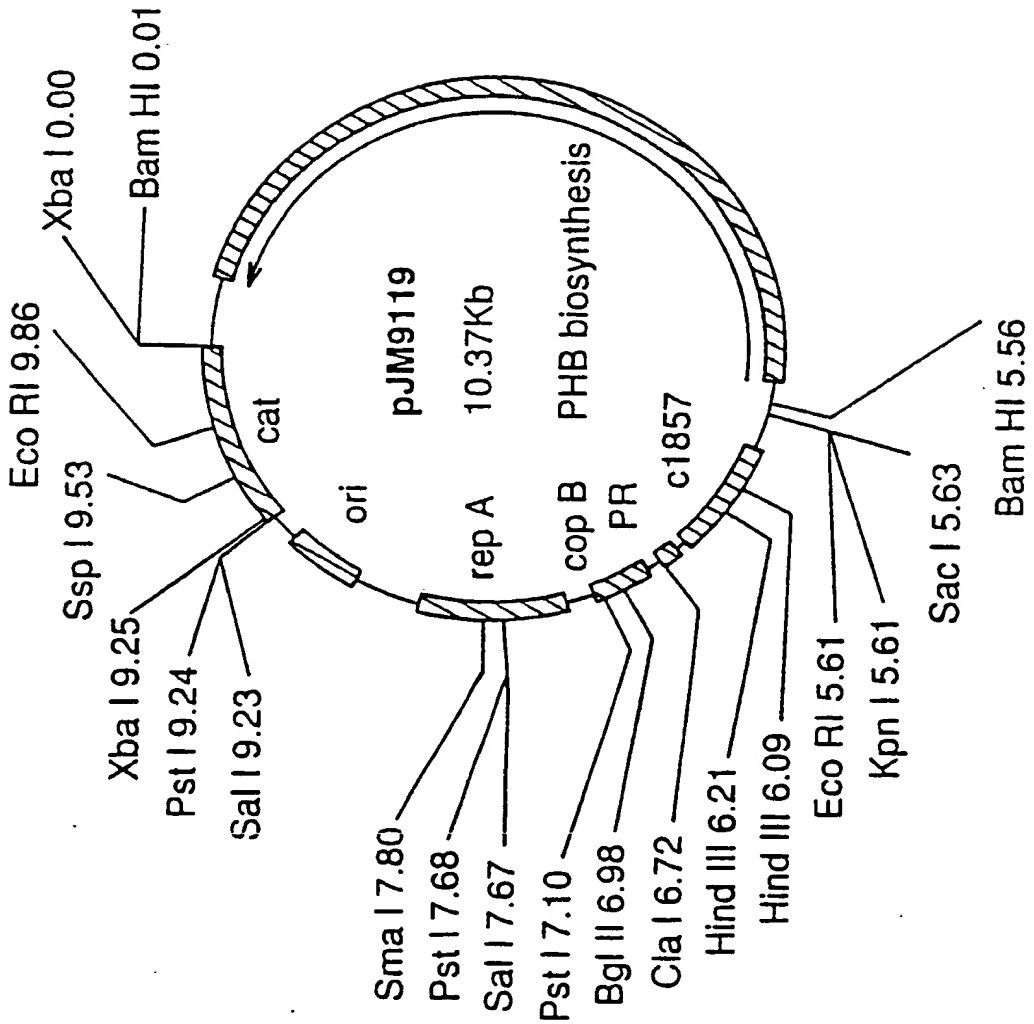


FIG.5B

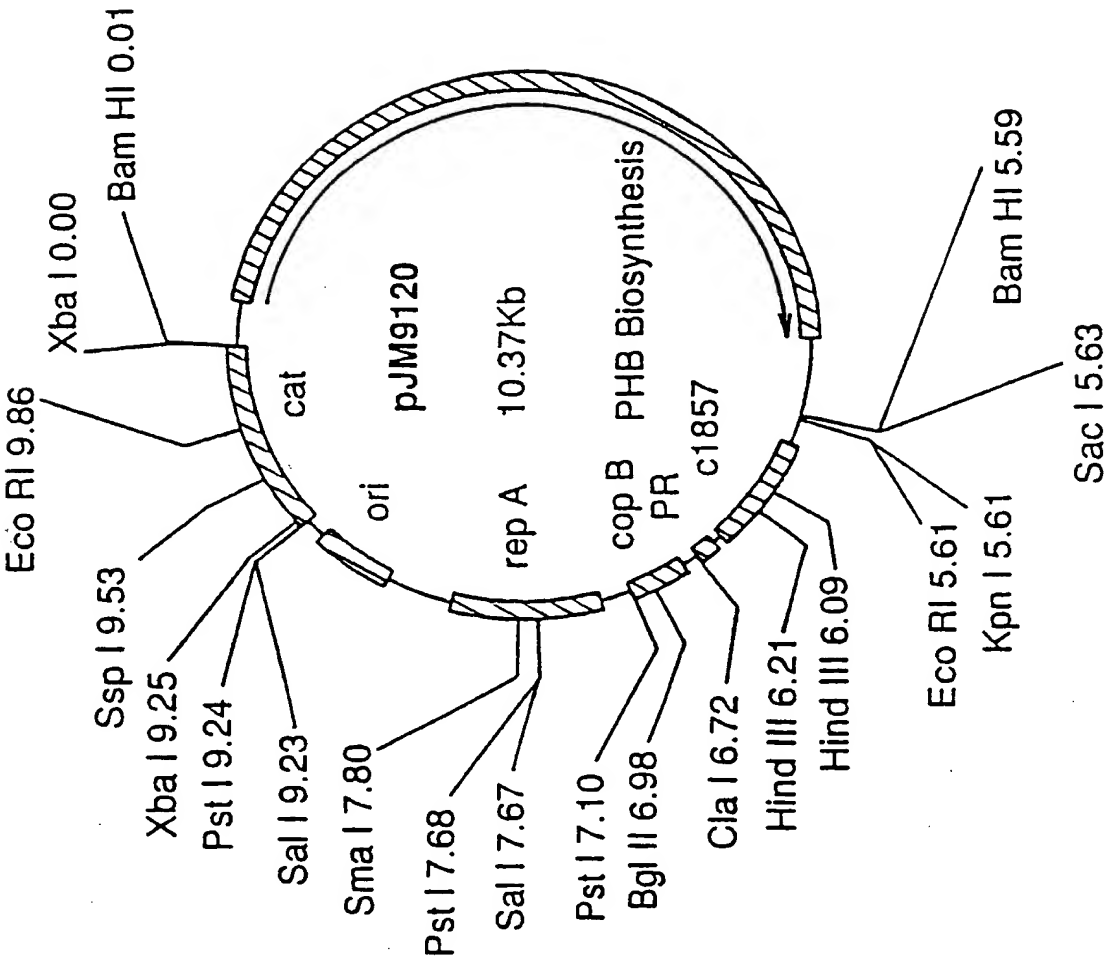
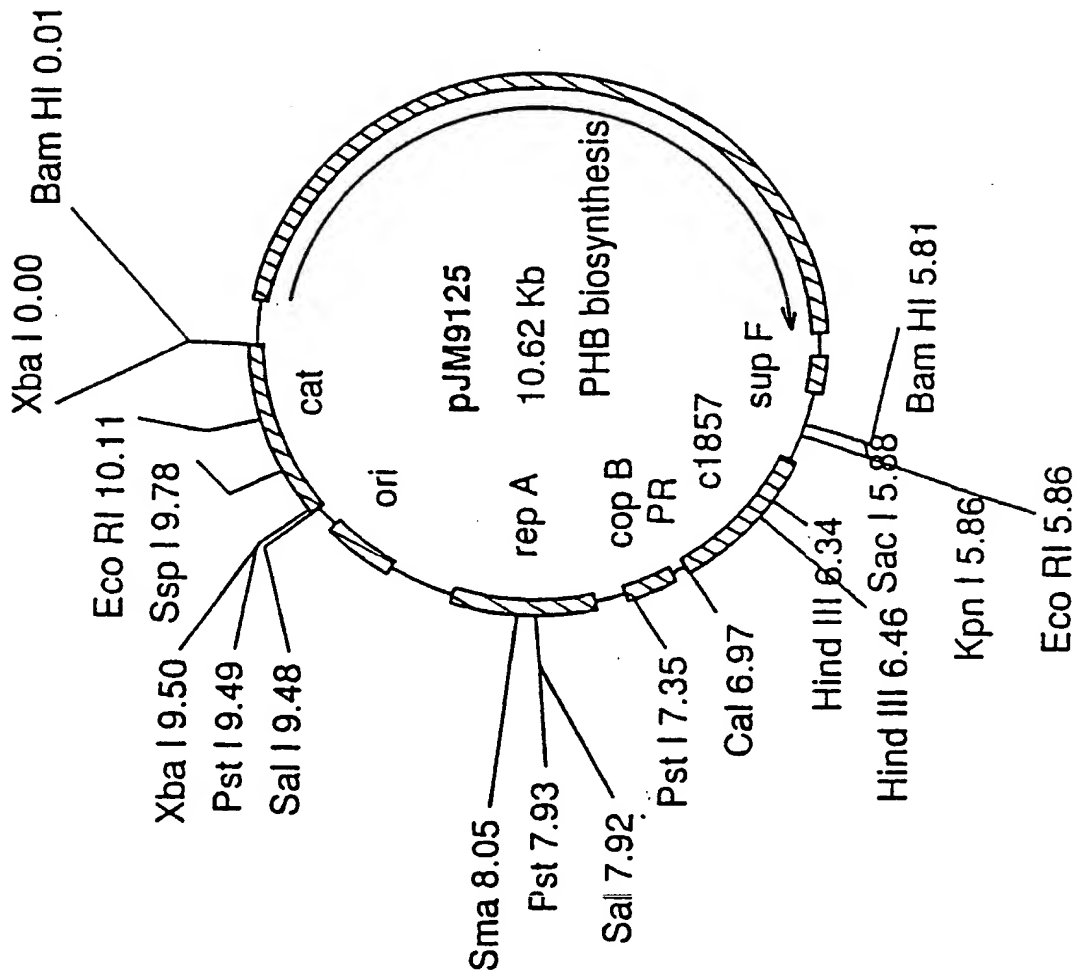


FIG.6A



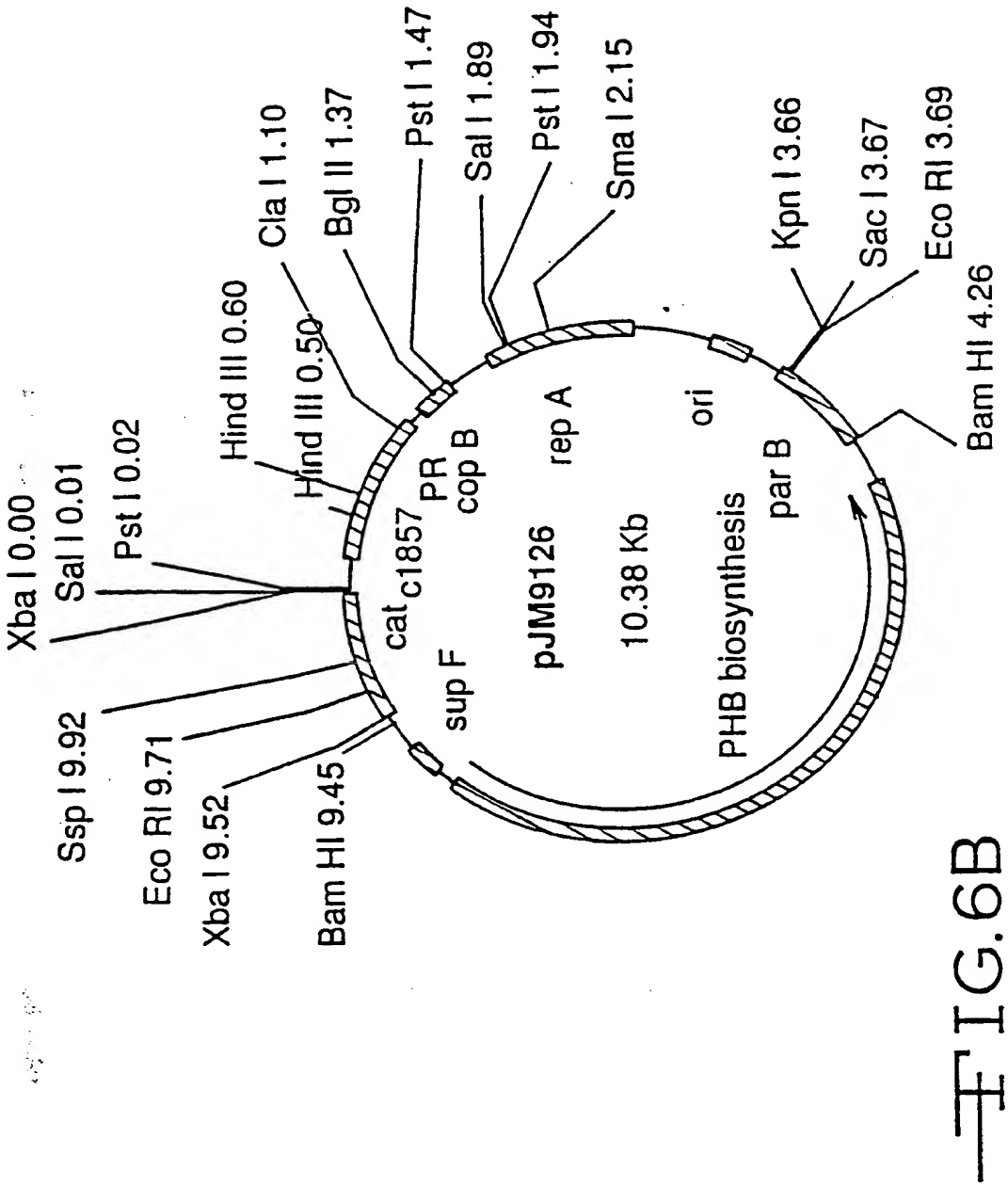


FIG.6B

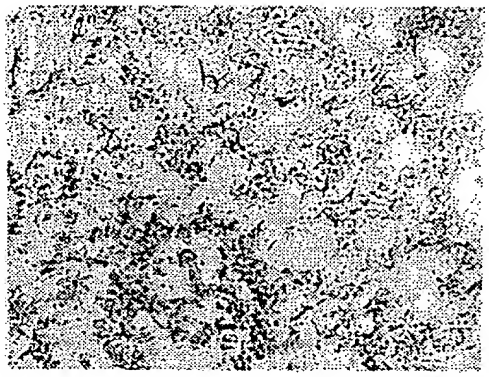


FIG. 7A

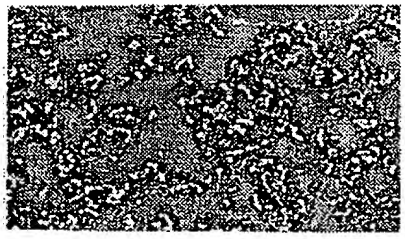


FIG. 7B

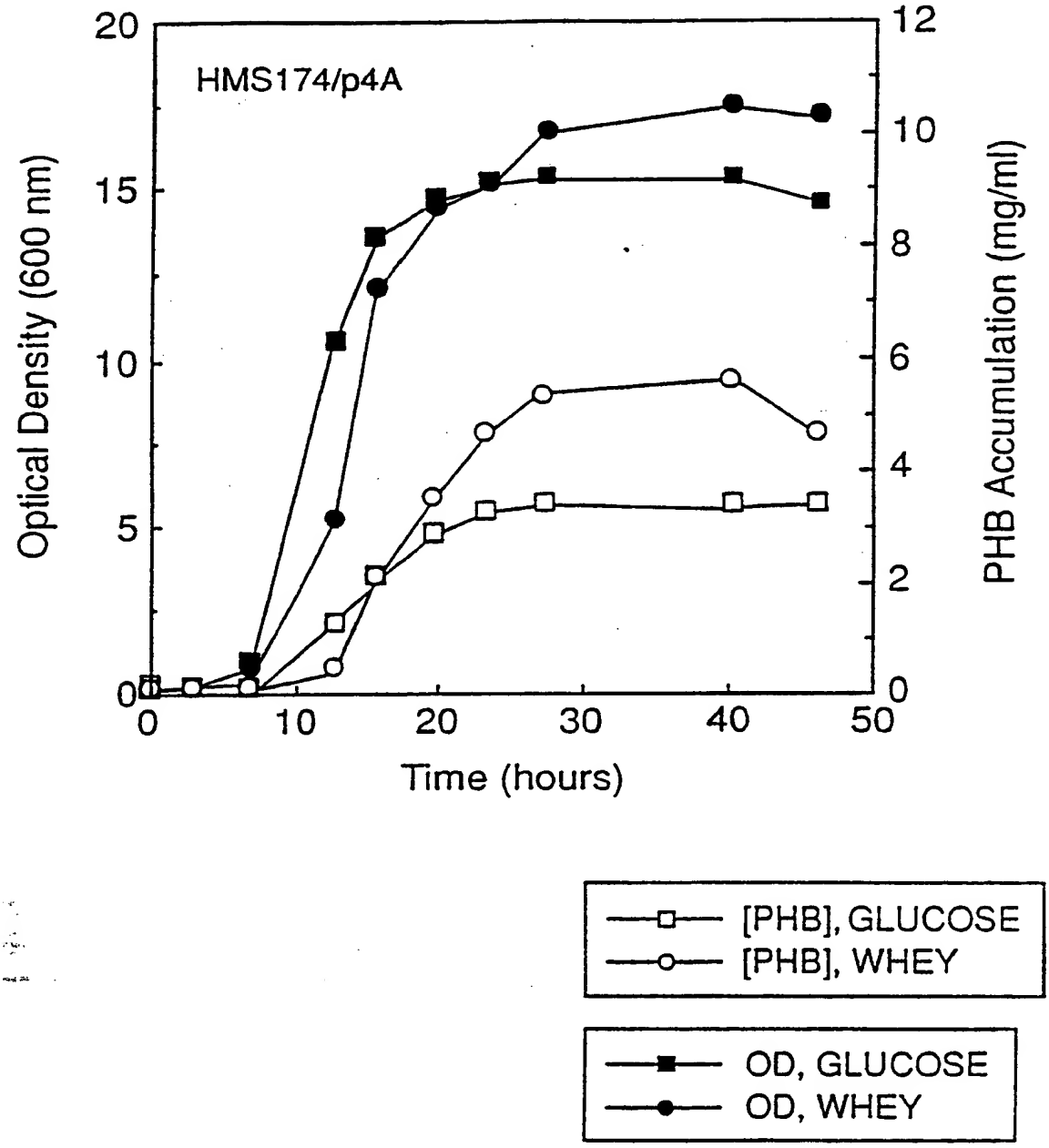


FIG.8A

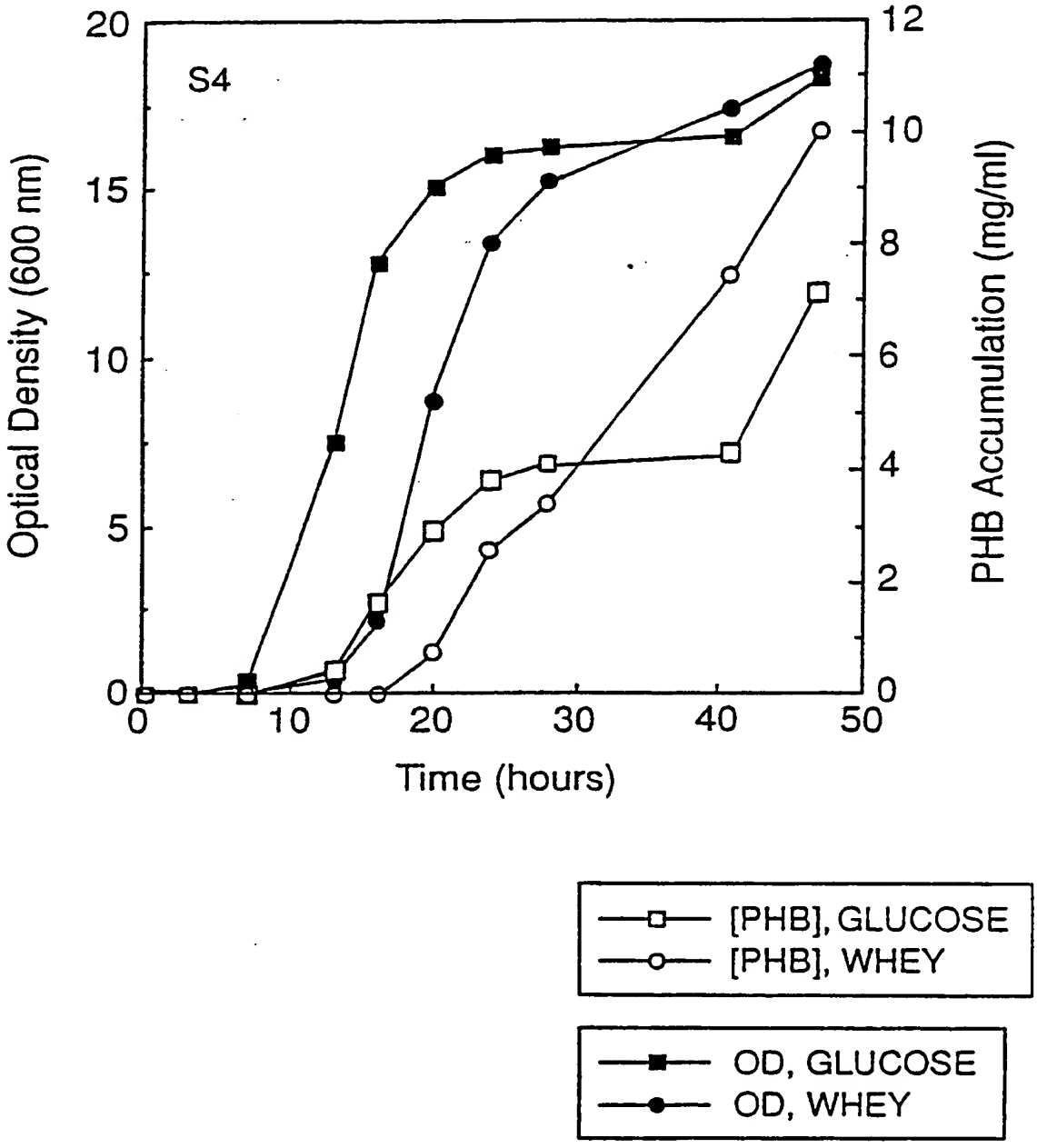


FIG.8B

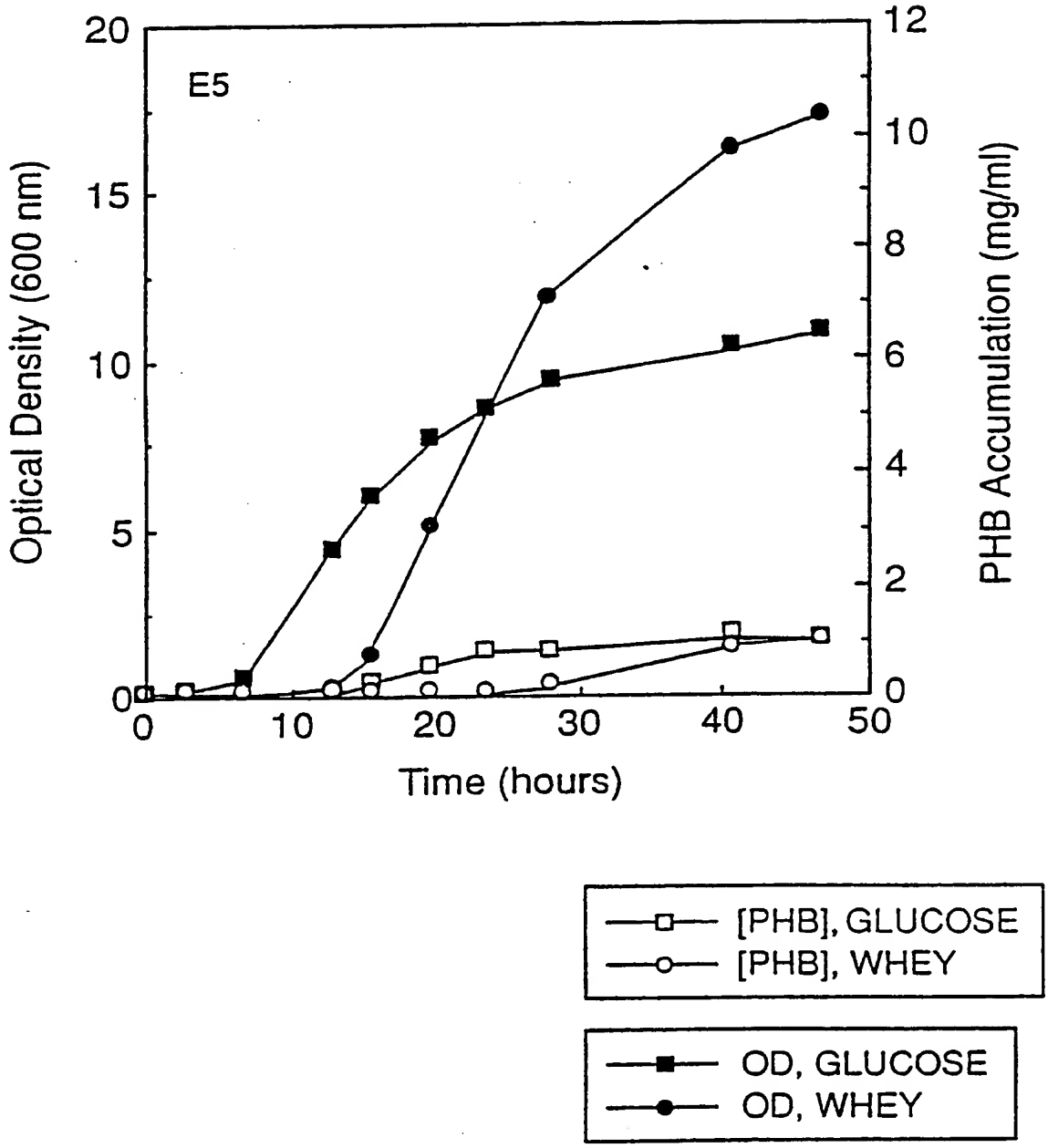


FIG. 8C

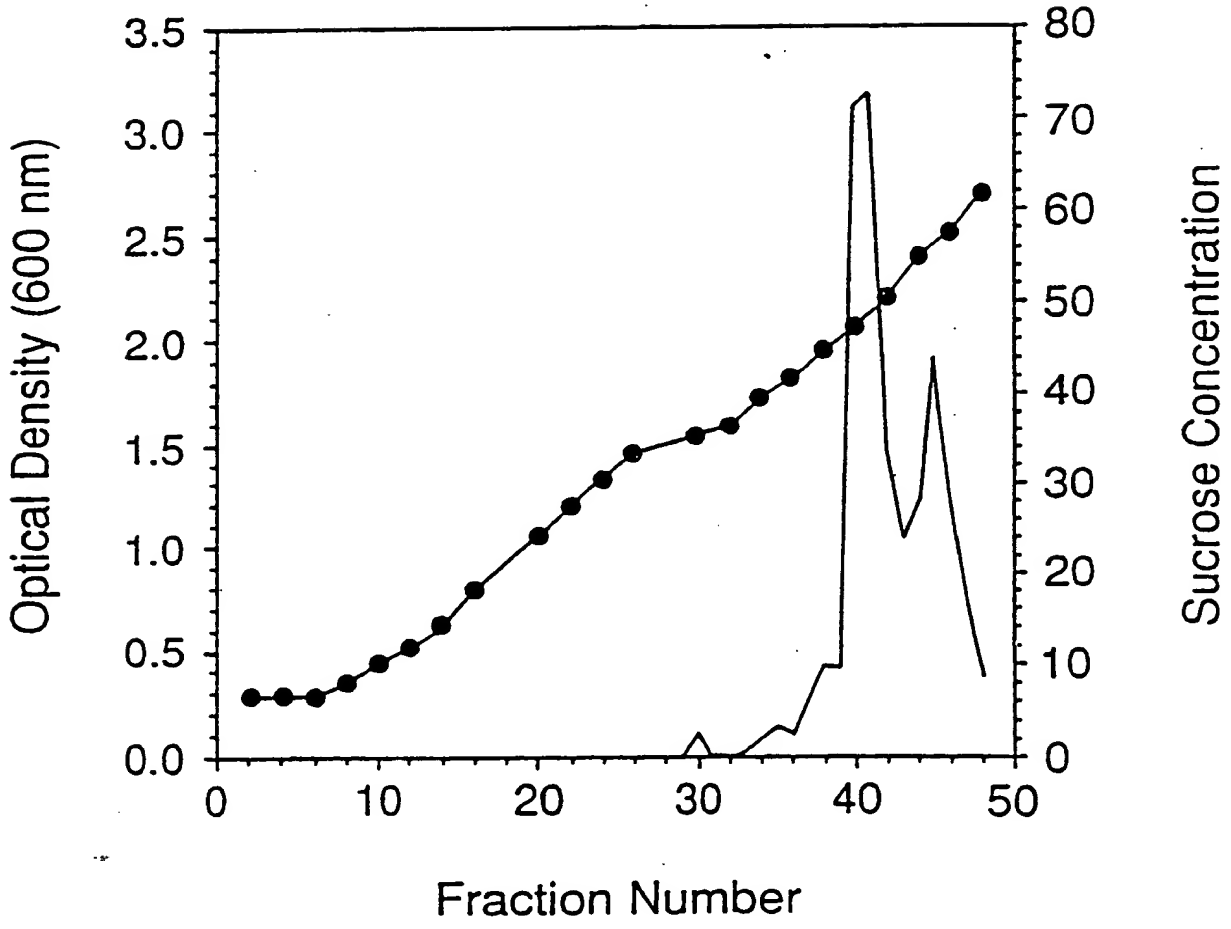


FIG. 9

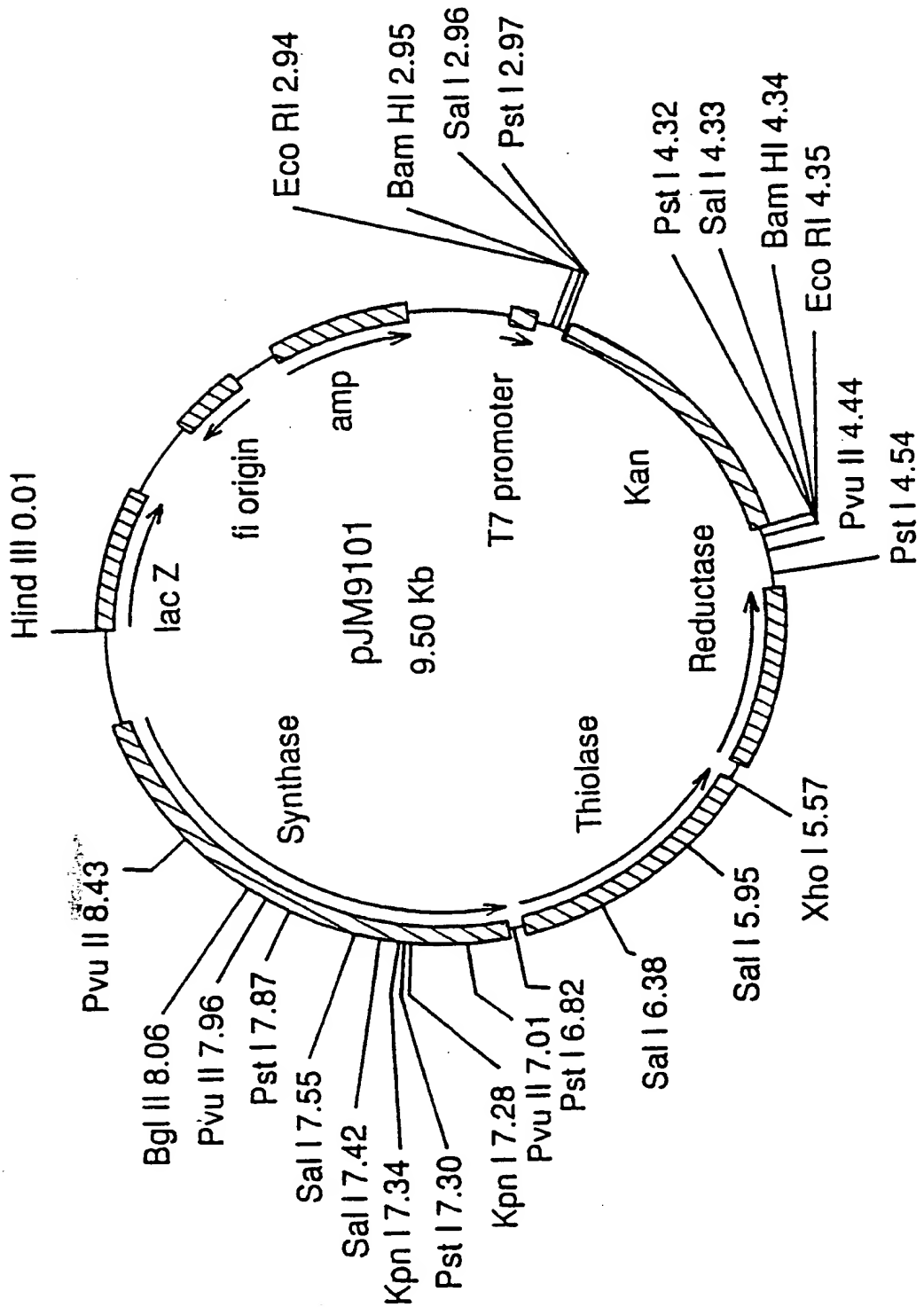


FIG. 10

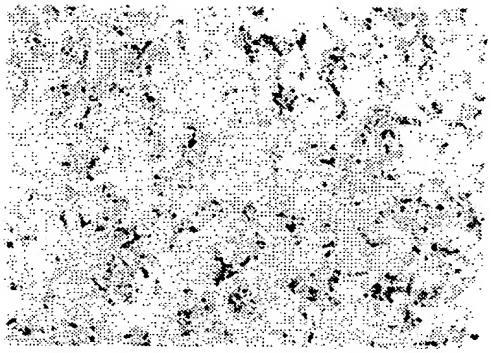


FIG. 11A

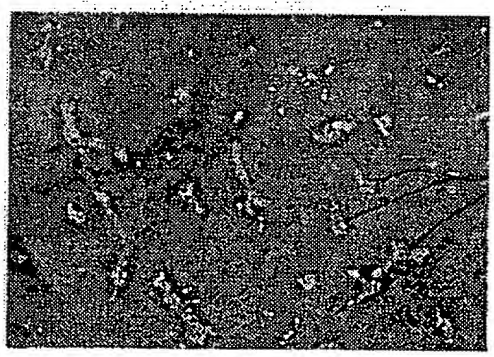


FIG. 11B

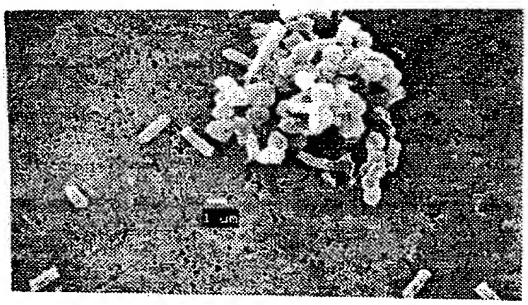


FIG 12A

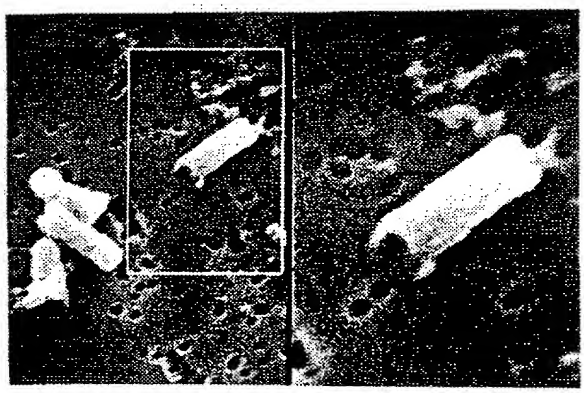


FIG. 12B

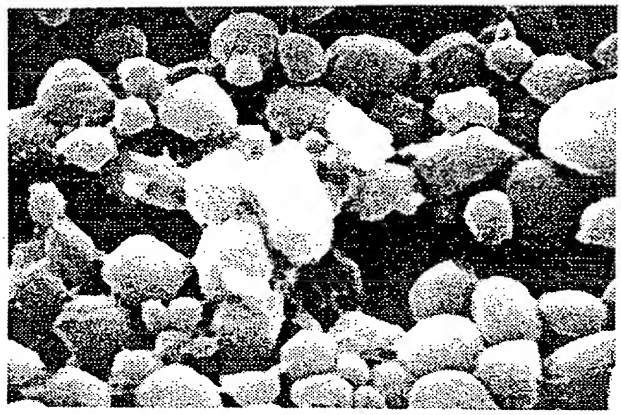


FIG. 12C

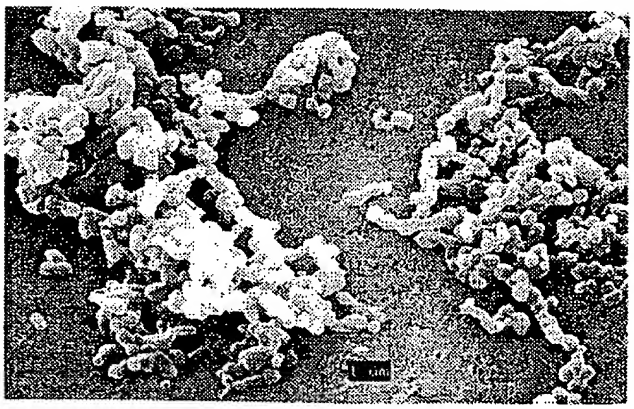


FIG. 13

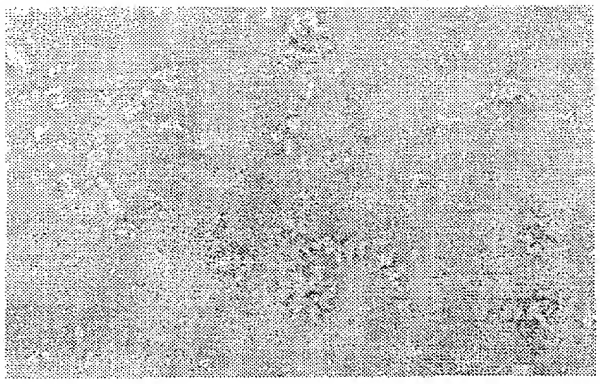


FIG. 14A

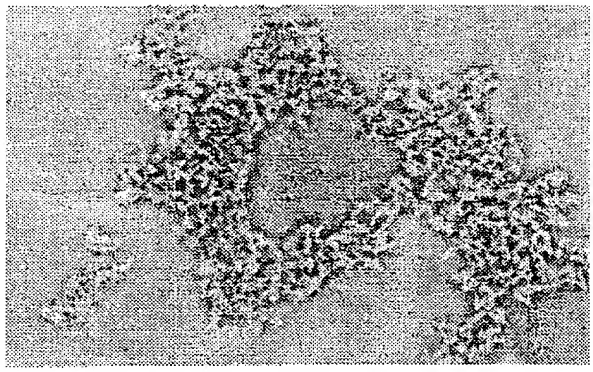


FIG. 14B

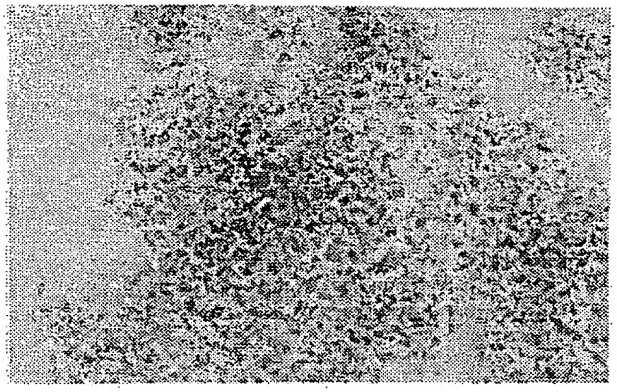


FIG. 14C

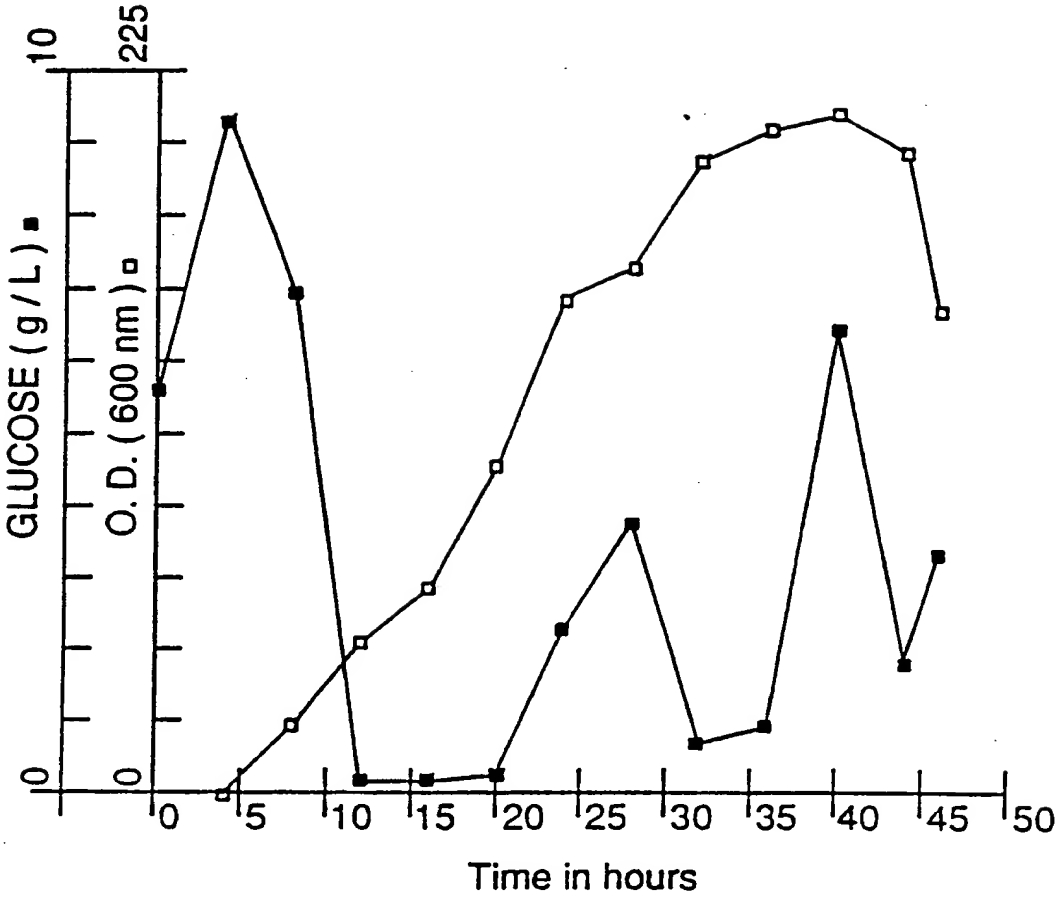


FIG. 15A

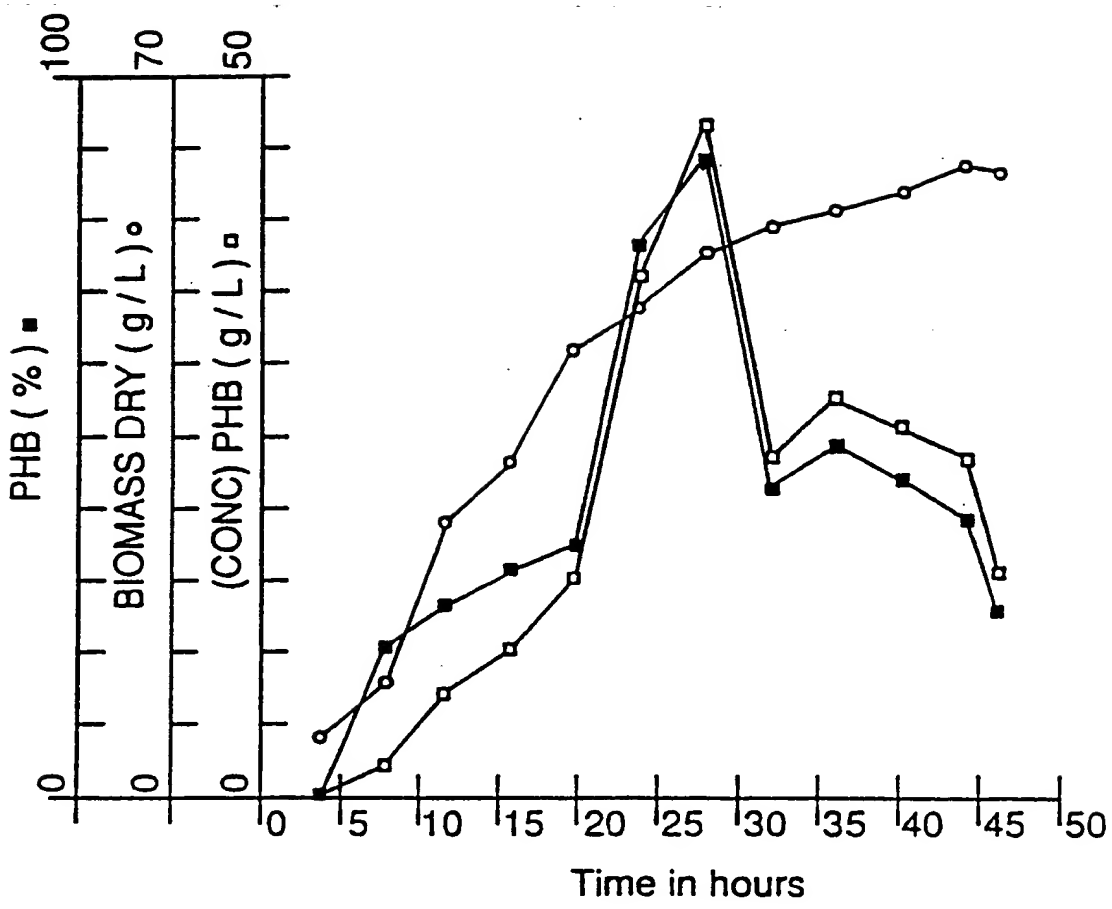

100
70
50
PHB (%) ■
BIOMASS DRY (g/L) ○
(CONC) PHB (g/L) □
0
0
0
0
5
10
15
20
25
30
35
40
45
50
Time in hours

FIG. 15B

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C12N 1/21, 1/06

A3

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(54) Title: METHOD FOR PRODUCTION AND RECOVERY OF POLY-β-HYDROXYBUTYRATE FROM TRANSFORMED *ESCHERICHIA COLI*

(57) Abstract

Poly-β-hydroxybutyrate is produced by providing a culture *Escherichia coli* bacterial host cells transformed by a DNA sequence coding for the biosynthetic pathway of poly-β-hydroxybutyrate and a DNA sequence coding for the lysozyme gene; growing the culture and obtaining expression of the poly-β-hydroxybutyrate biosynthetic pathway and the lysozyme gene in each *Escherichia coli* bacterial host cell; lysing the *Escherichia coli* bacterial host cells and collecting the poly-β-hydroxybutyrate. An *Escherichia coli* HMS174(p4A [BstB], pLysS) deposited with the American Type Culture Collection under Accession No. 69001, comprising a plasmid containing a biosynthetic pathway coding for poly-β-hydroxybutyrate and a plasmid containing a lysozyme gene is disclosed.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/05187

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/52 C 12 N 15/56 C 12 P 7/62
C 12 N 1/21 C 12 N 1/06

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 P C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	WO,A,9118993 (CENTER FOR INNOVATIVE TECHNOLOGY) 12 December 1991 cited in the application see page 3, line 10 - line 35 ---	1,5-8, 16,17 21
Y	EP,A,0155189 (GENENTECH, INC.) 18 September 1985 see page 1, line 5 - line 10; claims 1-9 ---	1,5-8, 16,17
A	DE,A,4003827 (PCD PETROCHEMIS DANUBIA DEUTSCHLAND GMBH) 22 August 1991 see column 1, line 3 - line 18 see column 1, line 67 - column 2, line 48 --- -/-	1,4,7

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06-10-1993	21. 02. 94
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOTECHNOLOGY ADVANCES vol. 9, no. 2, 1991, OXFORD GB pages 217 - 240 SUSAN T.L. HARRISON 'Bacterial cell disruption: a key unit operation in the recovery of intracellular products' see page 217, paragraph 1 see page 231, last paragraph - page 232, paragraph 1 see page 233, paragraph 1 ---	1
P,X	FEMS MICROBIOLOGY REVIEWS vol. 103, 1992, pages 231 - 236 STACY FIDLER ET AL. 'Polyhydroxyalkanoate production in recombinant Escherichia coli' see page 231, left column, paragraph 1 see page 232, left column, paragraph 1 see page 233, left column, paragraph 2 -----	1-4,7-9 ,13,15-17,21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05187

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.- claims 1-21
2.- claims 22-26

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-21

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9305187 SA 75655

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/02/94
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9118993	12-12-91	AU-A- 7996291	31-12-91
		EP-A- 0535065	07-04-93
EP-A- 0155189	18-09-85	JP-A- 60221077	05-11-85
DE-A- 4003827	22-08-91	None	

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82